SUBSTANCE P ACTIVATES CANCER PROMOTING PATHWAYS IN HEAD AND NECK CANCERS

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ABSTRACT

Substance P Activates Cancer Promoting Pathways in Head and Neck Cancers

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Head and neck cancer (HNC) is comprised of a multitude of malignancies that take place in the oral cavity. Neuropeptides such as Substance P (SP) are important in causing inflammation, proliferation and migration in different types of cell lines, but the precise effects and mechanisms are not yet elucidated in HNCs. Neuropeptide Substance P, along with its primary receptor, neurokinin-1 receptor (NK-1R), strongly affect the tumor microenvironment. In this study, the effects of SP, at a concentration of 100nM, was observed on HNCs including FaDu, Detroit 562 and SCC-9 cell lines. The inhibition of SP's most influential receptor NK-1R was then blocked using the drug L-703606 at a concentration of 1μM, and the resulting effects were measured. By utilizing an XTT Proliferation Assay, SP was found to significantly increase cell proliferation in all HNC cell lines tested, at the starting concentration of 100nM and throughout the increase to 10μM. SP also notably upregulated the expression of various cytokines, cytokine receptors, chemokines, chemokine receptors, MMP genes and EMT inducing

genes, while the NK-1R inhibitor significantly decreased these genes, which was evaluated via RT-PCR. Furthermore, SP induced the migration rate of HNC cell lines at a concentration of 100nM and was selectively inhibited by the NK-1R receptor at a concentration of 1μM, which was evaluated via migration assay and scratch wound assay. The nuclear translocation of NF-κB was also significantly increased in response to SP in immunofluorescence studies, and decreased when treated with NK-1R inhibitor. The effects of SP at 100nM significantly increased cellular inflammation, proliferation and migration of various HNCs, and its effects were inhibited by the drug L-703606, by blocking the NK-1R receptor.

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NOMENCLATURE

CCR	C-C motif chemokine receptor
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
EMT	Epithelial to mesenchymal transition
HNC	Head and neck cancer
IL	Interleukin
IL1β	Interleukin 1 beta
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteases
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK-1R	Neurokinin-1 receptor
OSCC	Oral squamous cell carcinoma
PD1	Programmed cell death protein 1
PDL1	Programmed death ligand 1
PTCHD	Patched domain-containing protein
SCC9	Squamous cell carcinoma 9
SHH	Sonic hedgehog
SNAI	Snail family transcriptional repressor
SP	Substance P
TWIST	Twist family BHLH transcription Ffactor
VEGF	Vascular endothelial growth factor

CHAPTER I

INTRODUCTION

Head and neck cancer is the 6th most common cancer worldwide and accounts for approximately 6% of cancer incidences, with 650,000 new cancer cases every year. [1] Head and neck cancer comprises primary tumor sites in the oral cavity, pharynx, larynx, paranasal sinuses and nasal cavity. HNC is known to be frequently recurrent and primarily metastasizes to the lungs, with the percentage of clinical metastasis ranging from 4-26%. Patients that have developed distant metastasis with HNC have a poor prognosis, even after attempted treatment [2].

Substance P is an 11 amino acid neuropeptide that is found in the central, peripheral, and enteric nervous systems, and the sequence for deriving SP is found on the preprotachykinin A gene [3]. Neurokinin 1 Receptor, along with neurokinin 2 receptor and neurokinin 3 receptor, are known to bind to SP and exert pro inflammatory effects [4]. The coupling of SP and NK-1R regulates significant inflammatory pathways such as the MAPK pathway and the EMT Pathway [5]. It was previously observed that the activation of NK-1R by SP, compared to the other two receptors, significantly increased the phosphorylation of myosin light chain 20, p38 mitogen associated protein kinase, and extracellular signal regulated kinase in the inflammatory and contractile MAPK pathways [6]. SP concentrations are elevated in inflamed conditions and play a significant role in tumor progression [7, 8]. Further, it has been studied that expression levels of NK-1R in tumor cells are correlated with the degree of malignancy of those tumor cells [9]. The rampant expression of the NK-1R and neurokinin 2 receptor has been identified in various cancer disease states such as breast cancer [10]. The coupling of substance P and NK-1R also has

effects on proliferation and migration of pancreatic cancer cells. Researchers previously identified that NK-1R was overexpressed in pancreatic cancer cells. SP plays an important role in the development of pancreatic cancer metastasis, and the inhibition of SP binding to NK-1R is an innovative method for the treatment of pancreatic cancer [11]. Previous studies show that an increase in SP levels is directly associated with progression of oral squamous cell carcinoma (OSCC), and the expression of SP directly correlates with increased tumor stage of OSCC [12]. However, the specific molecular and cellular effects that SP has on HNCs has not yet been elucidated identified and its role in cancer activating pathways remain unclear. Furthermore, the efficacy of NK-1R inhibition by drug L-703606 remains to be proven in HNCs.

The primary objective of this study is to delineate the specific effects of the inflammatory neuropeptide SP and on inflammatory and metastasis related genes in HNCs. The proliferative and migratory effects of SP on HNCs were also investigated. Additionally, the role of the NK-1R inhibitor, L-703606, in abrogating these SP mediated effects in HNC was analyzed.

CHAPTER II

METHODS

Cell Culture and SP/NK-1R Inhibitor Treatment

HNC FaDu, Detroit 562 and SCC9 cell lines were obtained from American Tissue Culture Collection. FaDu and Detroit cell lines were grown in MEM media supplemented with 10% FBS, 400ng hydrocortisone, and 1% penicillin/streptomycin, while SCC9 cell line was grown in DMEM-F12 media supplemented with 10% FBS, 400ng hydrocortisone, and 1% penicillin⁄streptomycin. Cell lines were grown in T-25 or T-75 flasks and maintained in humidified incubators at 37°C and 5% CO_2. Substance P and NK-1R inhibitor or L-703,606 (Sigma, MO, USA) were aliquoted into 10,000μM aliquots. SP was serial diluted from 10,000μM to 100μM, 10μM and 100nM aliquots. NK-1R inhibitor was then serial diluted from 10,000μM to 100μM and then 1μM aliquots. Aliquots of Substance P and NK-1R inhibitor below 10,000μM were prepared as needed. Cells for respective treatments were placed in plain media 2 hours prior to NK-1R inhibitor treatment. NK-1R inhibitor was diluted into 1μM and placed in respective wells. Substance P was added 2 hours after NK-1R inhibitor treatment and mixed with a pipette, if added along with NK-1R inhibitor.

RNA Isolation and RT- PCR Analysis

Cell lines FaDu, Detroit and SCC9 were placed on 12 well plates, grown to at least 90% confluency and treated with 100nM SP/1μM NK-1R inhibitor as described above. Total RNA was lysed and isolated using the Purelink RNA mini kit (Thermo Fisher Scientific, MA, USA) and according to manufacturer's protocol. A NanoDrop machine (NanoDrop Technologies, Wilmington, DE) was used to measure the quantity and quality of the isolated RNA. Total RNA

was then made into cDNA by using Maxima™ H Minus cDNA Synthesis Master Mix (Thermo scientific, MA, USA). Cytokine, chemokine, MMP and EMT inducing gene primers were created using SYBR Green (Applied Biosystems, CA, USA) and Real Time PCR was performed in a real time thermal cycler (ABI Prism 7900HT sequence detection system). All reactions had an N=3 and were compared to RPL19 as the reference gene. Data analysis was done on Microsoft Excel and GraphPad Prism, with Two-way ANOVA, followed by Fisher LSD for multiple comparisons.

XTT Cell Proliferation Assay

FaDu, Detroit and SCC9 cell lines were placed in 96 well plates and grown to reach 70% confluency. Cells were then washed with DPBS and placed in plain media 2 hours prior to SP treatment, which varied from 100nM-10μM concentrations. XTT Cell Proliferation Assay Kit (Trevigen, MD, USA) manufacture protocol was followed. 24 hours after SP treatment, media was removed and 100μL of DPBS was added to each well. XTT working reagent was prepared and 50μL of working reagent was added to each well. The 96 well plate was then incubated for 30 minutes and absorbance was measured at 490nm with a reference wavelength of 630nm, using Spectramax 650 (Molecular devices, CA, USA). Absorbance was measured for 1 hr, 1.5 hr, 2 hr and 3 hr time points and cells were incubated in between time points.

Boyden Chamber Assay

HNC cells (SCC9), was plated on 6 well plates, grown to at least 90% confluency and treated with 100nM SP/1μM NK1R inhibitor as described above. Cells were then trypsinized from the plates and $1x10^5$ cells were placed on top of 8.0-μm pore PTE standing inserts (Millipore, MA, USA). Inserts were placed in 24 well plates and respective complete media was placed in the lower chambers. Cells were allowed to migrate to the bottom of the insert for 24

hours. The inserts were then washed with DPBS, fixed with ice cold methanol for 1 hour and stained with 0.5% crystal violet for 1 hour. A Nikon inverted microscope was used to capture 10x magnified images of the inserts. Five different fields were imaged, and the quantity of cells migrated was quantified using the Image J software, version 1.52a (National Institute of Health)

Scratch Wound Assay

One of the cell lines was randomly selected (Detroit), plated on a 12 well plate and grown to at least 90% confluency. Respective wells were primed with 1μM NK-1R inhibitor for 2 hours. Pipette tips were used to make 3 scratches in each well, with 1 in upper field, 1 in middle field and 1 in lower field. 100nM SP was immediately added to respective wells after scratches were made. Cells were allowed to sit in complete media for 24 hours, and 4x images were captured of each scratch at 0, 12 and 24 hours using an inverted microscope. Scratch wound closure or migration rate was determined using ImageJ. Migration rate was expressed as average migration rate in μm/hr.

Immunofluorescence

HNC cells (FaDu), were plated on coverslips and grown to at least 70% confluency. Respective coverslips were untreated, treated with 100nM SP or treated with 1μM NK-1R inhibitor + 100nM SP as described above. After 24 hours of treatment, coverslips were fixed with 2% paraformaldehyde and permeabilized with ice cold methanol. Immediately afterwards, NF-κB primary antibody was placed on cover slips for 2 hours. Cover slips were then incubated with Goat anti-rabbit secondary antibody (AlexaFlour® 488 Green) for 1 hour in the dark and washed several times. Using Prolong Gold anti-fade solution, the coverslips were mounted onto glass slides and allowed to cure overnight. 20x Fluorescent images were captured using a fluorescent microscope, at a scale of 100μM. Images were captured in green fluorescence for

NF-κB and blue fluorescence for DAPI nucleus staining, and both of these images were merged. The percentage of NF-κB fluorescent intensity was calculated and plotted for each treatment group.

Statistical Analysis

All experiments were carried out with at least an N = 3. Values were averaged for each treatment group and statistically compared against each other. Using GraphPad Prism, Two-way ANOVA was performed, followed by Fisher LSD to measure significance of comparisons. Values are represented as mean ± SE on each graph. *, **, ***, **** or #, ##, ### and #### indicates $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, $p \leq 0.0001$, as compared to control or SP 100nM, respectively.

CHAPTER III

RESULTS

Substance P Upregulates the Expression of Tumor Promoting Genes

In previous studies, SP was found to be associated with increased levels of inflammation, proliferation and migration in cancer; however, the specific inflammatory and metastasis related markers that SP induces has yet to be delineated in HNCs. Using real time PCR, the expression of various genes was carried out to determine the effect that SP has on HNC cell lines. It was noticed that 100nM Substance P caused a significant increase in the expression of various inflammatory cytokines, cytokine receptors, chemokines, chemokine receptors, metalloproteinase (MMP) and epithelial to mesenchymal transition (EMT) inducing genes in HNC cell lines. Further, 1μM NK-1R inhibitor significantly reduced the effects of 100nM SP in many of the previously induced genes, with the exception of a few. The following PCR results were observed and are shown in Fig. 1, 2 and 3 (in the Appendix) for FaDu, Detroit and SCC9, respectively.

FaDu PCR Analysis

After treatment with 100nM SP for 24 hours, FaDu cells showed a significant increase in expression of certain genes compared to the untreated control, including cytokines – MCP1 and PD1 (Fig. 1A), chemokines – CXCL9, CCL19 and CCL21 (Fig. 1A), cytokine receptors – CCR2, CCR3, CCR4, CCR5 and CCR9 (Fig. 1B), chemokine receptors – CXCR1, CXCR3, CXCR3B, CXC3R1 and CXCR4 (Fig. 1B), MMP genes – MMP2, MMP7, MMP11 and MMP12 (Fig. 1C) and EMT genes – PTCHD, GLI and TWIST1 (Fig. 1C). The SP treatment group was

then compared with the 1μM NK-1R inhibitor + 100nM SP group, which showed significant decreased expression in all previously mentioned genes (Fig. 1A-C).

Detroit PCR Analysis

Furthermore, treatment of Detroit cells with 100nM SP significantly upregulated the expression of similar tumor promoting genes, including cytokines - IL1β, MCP1, IL6 and PD1 (Fig. 2A), chemokines - CXCL1, CXCL9, CCL19 and CCL21 (Fig. 2A), cytokine receptors - CCR3, CCR5, CCR7, CCR9 and CCR10 (Fig. 2B), chemokine receptors - CXCR1, CXCR3, CXCR3B, CXC3R1 CXCR4 and CXCR6 (Fig. 2B), MMP genes - MMP12 and MMP21 (Fig. 2C) and EMT genes - PTCHD, TWIST1 and SHH (Fig. 2C), though there was no significant increase in MMP genes. Detroit cells were then primed with 1μM NK-1R inhibitor prior to 100nM SP treatment, and this group showed significant downregulation of the formerly mentioned genes (Fig. 2A-C) when compared with the 100nM SP treatment group. However, CXCR1, CXCR3B and MMP21 genes did not show significant decrease when comparing NK-1R inhibitor + SP and SP treatment groups.

SCC9 PCR Analysis

To further support the hypothesis, SCC9 cells were treated with 100nM SP and showed significant induction of specific proinflammatory and metastatic promoting genes, including cytokines - PD1 (Fig. 3A), chemokines - CXCL1, CXCL5, CXCL9, CXCL10 and CCL1 (Fig. 3A), cytokine receptors - CCR4, CCR5, CCR6 and CCR10 (Fig. 3B), chemokine receptors - CXCR1, CXCR3B, CXC3R1, CXCR4 and CXCR6 (Fig. 3B), EMT inducing genes: PTCHD, TWIST1 and TWIST2 (Fig. 3C). Upon treatment of SCC9 cells with 1μM NK-1R inhibitor prior to SP treatment, the previously induced genes were seen to be significantly inhibited, compared to the 100nM SP treatment group. However, CXCL10, CXCR3B, CXCR4 and PTCHD

expression was not inhibited in the NK-1R inhibitor + SP group, compared to the SP treatment group.

Substance P Promotes the Proliferation of HNC Cell Lines

Cellular proliferation is known to play a vital role in the growth and invasion of cancer cells, as noted in previous studies. SP was shown to increase cellular proliferation of colorectal cancer [8]; however, the effect of SP on cellular proliferation of HNC has yet to be observed. XTT cell proliferation was carried out and all 3 HNC cell lines, FaDu, Detroit and SCC9, were treated with various concentrations of SP (100nM - 10μM), data was collected with a $n \geq 3$ and plotted (Fig. 4). It was observed that all 3 cell lines had increased % cellular proliferation in response to SP treatment. Significance was noted in the lowest concerntration of 100nM in the 3 cell lines, with 15% increase in FaDu (Fig. 4A), 90% increase in Detroit (Fig. 4B) and 16% increase in SCC9 (Fig. 4C). Significant increase in cell proliferation was noted with all SP treatment, including 100nM, 400nM, 600nM, 800nM, 1μM and 10μM concentrations.

Substance P Induces Migration of HNC Cells

Migration of cancer cells is directly related to increased cancer metastasis and invasion of cancer cells in surrounding tissues and is a prerequisite for distant dissemination. It has been noted that SP induces migration of pancreatic cancer cells, and also plays key roles in wound healing and wound closure [11,13]. However, the effect of SP and the efficacy of NK-1R inhibitor, L-703606, on HNC migration has not yet been observed. To measure the migratory effect of HNC, boyden chamber assay and the scratch wound assay was carried out for SCC-9 and Detroit cell lines. In both methods, 100nM SP treatment group had significantly increased the migration rate of the HNC cells, while 1μM NK-1R inhibitor significantly abrogated the effects of SP.

SCC9 Boyden Chamber

Boyden chamber assay was carried out as described earlier in the methods, SCC9 cells were treated with 100nM SP, which showed a significant increase in the number of cells migrated towards complete media by 40% (Fig. 5), compared to the untreated cells. The cells that were treated with 1μM NK-1R inhibitor prior to SP treatment showed a significant decrease in cell migration, compared to the SP treatment group. Further, the treatment group with only 1μM NK-1R inhibitor treatment did not show significant deviation in cell count compared to the untreated control. These trends are obviously visible and shown in the captured images of the inserts (Fig. 5A). The migrated cells were quantified and plotted on a graph as well (Fig. 5B).

Detroit Scratch Wound

After treating Detroit cells with SP and NK-1R inhibitor appropriately and abiding by the scratch wound assay, the wound closure rate was monitored for 24 hours (Fig. 6). It was observed that Detroit cells treated with 100nM SP had increased wound closure at both 12 and 24 hours, compared to the untreated cells. Further, 1μM NK-1R significantly abrogated the effects of SP and did not have significant wound closure compared to the scratches treated with SP only. The treatment group with only 1μM NK-1R inhibitor did not have significant deviation from the untreated control group. The observed wound closure is directly related to the migration rate of cells and was measured appropriately. These trends are evidently seen in the images captured at the 12- and 24-hour time points (Fig. 6A). The migration rate was then measured in μM/hr and plotted (Fig. 6B).

Substance P Activates Nuclear Translocation of NF-κB and Promotes Inflammatory Pathways

NF-κB is a transcription factor that is central to the process of inflammation and is closely associated with increased proliferation, migration and metastasis of several human cancers by translocating to the nucleus of the cell and activating a cascade of inflammatory genes [17]. However, this effect has not been studied with SP in HNCs. Utilizing immunofluorescence techniques, we determined that SP treatment (100nM) significantly increases the nuclear translocation of NF-κB, compared to the untreated control. Furthermore, the 1μM NK-1R inhibitor pre-treatment significantly abrogated the effects of 100nM SP treatment, which can be seen by the decreased intensity of nuclear NF-κB. These results can be clearly visualized in the fluorescent images captured of the slides (Fig. 7A), which merges the nuclear DAPI stain and NF-κB stain. The intensity of NF-κB fluorescence in the nucleus was measured and plotted (Fig. 7B).

CHAPTER IV

CONCLUSION

The data presented in this study indicates that Substance P significantly increased cellular inflammation, proliferation, and migration of 3 different types of head and neck cancer cell lines. The results also demonstrate that the effects of Substance P were inhibited by the L-703606 drug through effective blockage of the NK-1R receptor.

The PCR results from Fig. 1 for the FaDu cells demonstrate that 100nM of Substance P resulted in significant increases in the expression of genes coding for the following genes: cytokines: MCP1 and PD1 (Fig. 1A), chemokines: CXCL9, CCL19, and CCL21 (Fig. 1A), cytokine receptors: CCR2, CCR3, CCR4, CCR5, and CCR9 (Fig. 1B), chemokine receptors: CXCR1, CXCR3, CXCR3B, CXC3R1,and CXCR4, MMP (Fig. 1B), MMP genes: MMP2, MMP7, MMP11, and MMP12 (Fig. 1C), and EMT genes: PYCHD, GLI and TWIST1 (Fig. 1C). The expression of these same genes was significantly decreased in the group that was treated with 1μM NK-1R inhibitor and 100nM SP. This data plays a key role towards the hypothesis because cancer progression involves overexpression of MMPs, which in turn mediate many of the changes in the tumor-microenvironment associated with angiogenic or lymphangiogenic processes [14]. For example, MMP9 modulates bioavailability of vascular endothelial growth factor and may directly affect tumor-induced lymphangiogenesis [14]. In addition, MMP7 also promotes tumor migration and invasion [15]. Furthermore, the invasion of cancer cells to different tissues involves MMPs, which work to degrade the local extracellular matrices and allow tumor cells to migrate into surrounding areas [22,23]. From the results of the FaDu PCR it can be stated that the effects of SP were significantly reduced in FaDu cells that were exposed to

the 1μM NK-1R inhibitor treatment. This supports the hypothesis because the FaDu cell group that was treated with 100nM of SP experienced significantly higher expressions of the discussed genes (Fig. 1A-C) and when this group was compared to the group of FaDu cells treated with 100nM of SP and 1μM NK-1R inhibitor, the group that was treated with 100nM SP and 1μM NK-1R inhibitor displayed a significant decrease in expression of the same genes (Fig. 1A-C). This indicates successful abrogation of the NK-1R receptor in the FaDu cell line. From a physiological standpoint this would indicate a significantly reduced amount of inflammation.

The PCR results from Fig. 2 for the Detroit cells demonstrate that 100nM of Substance P resulted in significant upregulation in the expression of tumor promoting genes such as: cytokines: IL1β, MCP1, IL6 and PD1 (Fig. 2A), chemokines: CXCL1, CXCL9, CCL19 and CCL21 (Fig. 2A), cytokine receptors: CCR3, CCR5, CCR7, CCR9 and CCR10 (Fig. 2B), Chemokine receptors: CXCR1, CXCR3, CXCR3B, CXC3R1 CXCR4 and CXCR6 (Fig. 2B), MMP genes: MMP12 and MMP21 (Fig. 2C) and finally EMT genes: PTCHD, TWIST1 and SHH (Fig. 2C). Significant downregulation in the expression of these genes (Fig. 2A-C) were observed in the group of Detroit cells that were primed with 1μM NK-1R inhibitor before exposure to 100nM SP, with the exception of the following genes: CXCR1, CXCR3B and MMP21. Proinflammatory cytokines/chemokines can work to promote positive feedback regulation of NK-1R and SP, to further promote proliferation and inflammation by immune cells [19,20,21]. The data from these Detroit PCR experiments supports the hypothesis because the Detroit cells that were treated with the NK-1R inhibitor prior to exposure with 100nM SP displayed a significant decrease in expression of the genes in Fig. 2A-C (with the exception of CXCR1, CXCR3B and MMP21). This indicates successful blocking of the NK-1R receptor in

the Detroit cells. From a physiological view this would result in a significantly reduced amount of inflammation and tumor promotion.

The PCR results from Fig. 3 for the SCC9 cell line suggest that 100nM of Substance P demonstrated significant induction in the expression of proinflammatory genes as well as metastatic genes. There were significant increases in the expression of genes encoding for the following: cytokines: PD1 (Fig. 3A), chemokines: CXCL1, CXCL5, CXCL9, CXCL10, and CCL1 (Fig. 3A), cytokine receptors: CCR4, CCR5, CCR6, and CCR10 (Fig. 3B), and finally EMT inducing genes: PTCHD, TWIST1, and TWIST2 (Fig. 3C). There was significant reduction in the expression of these genes (Fig. 3A-C) in the group where the SCC9 cells were treated with 1μM NK-1R inhibitor prior to 100nM SP treatment, with the exception of the following genes: CXCL10, CXCR3B, CXCR4 and PTCHD. TWIST1 has specifically been noted to promote basement degradation via EMT, and play a role in invadopodia mediated matrix degradation [27]. The data from these SCC9 PCR experiments support the hypothesis because it displays that succesful inhibition of the NK-1R receptor by the 1μM NK-1R inhibitor showed significant decreases in the expression of the proinflammatory and metastatic genes. From a physiological stance it can be conjectured that there will be decreased inflammation and decreased metastasis due presence of the L-703606 NK-1R inhibitor.

Our data clearly shows that Substance P promotes proliferation of HNC cells, this is supported by the XTT assays. In the XTT assay for cellular proliferation it was seen that there was an increase in cellular proliferation in all three cell lines. The FaDu cell line (Fig. 4A) showed a 15% increase in proliferation when treated with 100nM SP, the Detroit cell line (Fig. 4B) showed a 90% increase in proliferation when treated with 100nM SP, and the SCC9 cell line (Fig. 4C) showed a 16% increase in proliferation when treated with 100nM SP. This data

supports the hypothesis because the significant proliferation of HNC cells is evident in the presence of SP. From a physiological perspective it can be discerned that in the presence of SP (no inhibitor) the HNC cells (from cell lines FaDu, SCC9, Detroit) will display increased cellular proliferation.

Results from the Boyden chamber assay supports a significant increase in the migration of HNC cells. This is because when the SCC9 cells were treated with 100nM SP, the cells increased migration (Fig. 5) towards complete media by 40%. It should also be noted that the SCC9 cells that were treated with 1μM NK-1R inhibitor before 100nM SP treatment showed significant decreased migration towards the complete media. Another observation is that SCC9 cells that were treated with the 1μM NK-1R inhibitor alone did not see any significant changes to migration towards complete media. These data are crucial for the evidence supporting the hypothesis because EMT is critical for early metastasis, is often induced by inflammation, and endows tumor cells with enhanced migratory and invasive properties [16]. Further, EMT activation precedes the downregulation of epithelial junction proteins, which assist in maintaining junctions and desmosomes, and ultimately leads to the loss of apical basal polarity. EMT genes allow tumor cells to migrate easily, without the hinderance of epithelial structure [25]. These changes in epithelial junctions also play a role in numerous downstream pathways, which may further promote EMT and migration [26]. These data from the Boyden chamber assay suggest that in presence of SP, HNC cells will migrate more and reduce the survival rate of patients. This supports the hypothesis because in the presence of SP, the SCC9 cells are migrating significantly more than the SCC9 cells that were not treated with the SP treatment.

Further, data from the scratch wound assay indicate that the presence of the 1μM NK-1R inhibitor along with the 100nM SP in Detroit cells reduced the closure rate. The Detroit cells that

were treated with only the 100nM SP showed a significantly faster closure rate when compared to that of the Detroit cells treated with 100nM SP and 1μM NK-1R inhibitor. This supports the hypothesis because it is indicating that the NK-1R inhibitor is successfully blocking the actions of the SP and NK-1R pathway. It is known that SP is involved in wound closure and healing [13] so it is justified that when the binding of SP to the NK-1R receptor is blocked, the wound healing response should be mitigated. This further reinforces that the NK-1R inhibitor reduced migration.

Another piece of evidence emerges from the Nuclear Translocation experiments of NF-κB. Activation of NF-κB nuclear translocation plays a role in MAPK activation in immune cells, further augmenting inflammation and the production of inflammatory signaling [17,18]. It was observed that the significant increase in the intensity of NF-κB in the FaDu cells treated with 100nM of SP when compared with untreated cells. When the FaDu cells were treated with the 1μM NK-1R prior to treatment with 100nM SP, there was a significant decrease in intensity of NF-κB. This data is relevant to the hypothesis because SP is able to activate chemokines, such as CCL2, CCL4 and CXCL2, through nuclear translocation of NF-κB, which ultimately attracts immune cells to inflammation sites [17,24]. Further, when 1 μM NK-1R inhibitor is present, there is less intensity of the NF-κB which leads to the conclusion that there will be less proliferation, migration, and metastasis of head and neck cancer cells.

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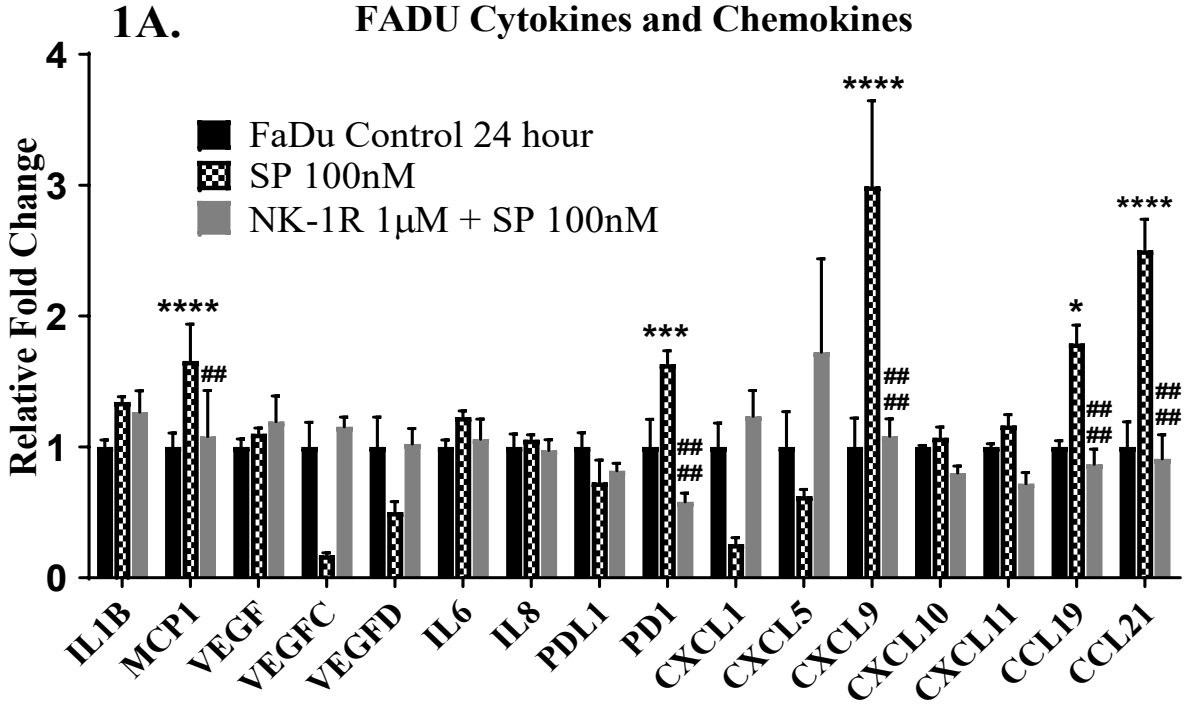
APPENDIX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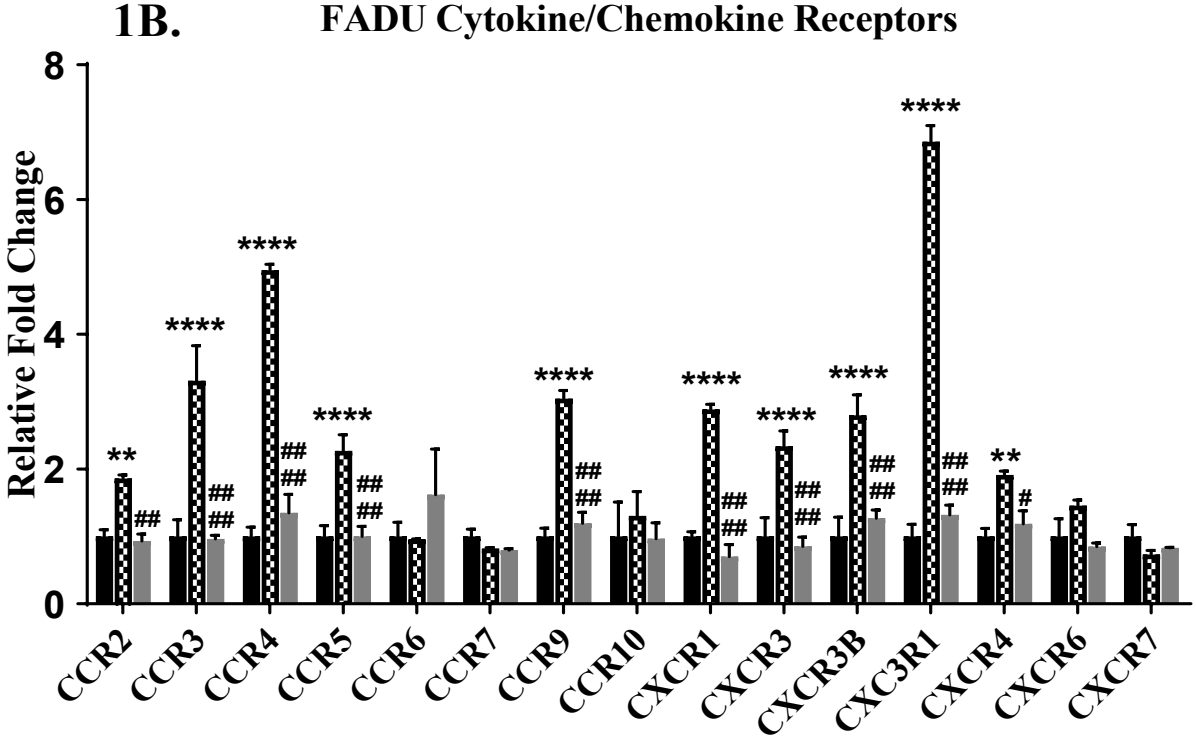
FADU Cytokines and Chemokines
FaDu Control 24 hour
SP 100nM
NK-1R 1μM + SP 100nM
Relative Fold Change
IL1B
MCP1
VEGF
VEGFC
VEGFD
IL6
IL8
PDL1
PD1
CXCL1
CXCL5
CXCL9
CXCL10
CXCL11
CCL19
CCL21


1B.
FADU Cytokine/Chemokine Receptors
Relative Fold Change
CCR2
CCR3
CCR4
CCR5
CCR6
CCR7
CCR9
CCR10
CXCR1
CXCR3
CXCR3B
CXC3R1
CXCR4
CXCR6
CXCR7

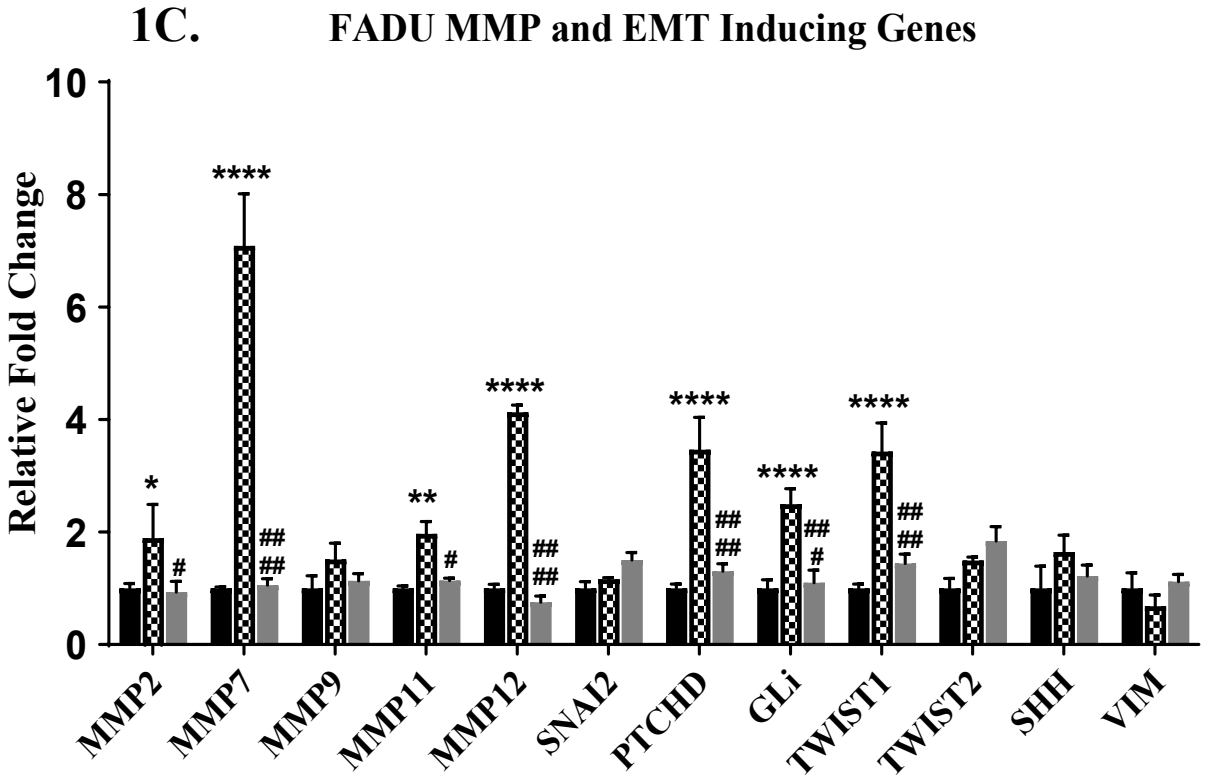
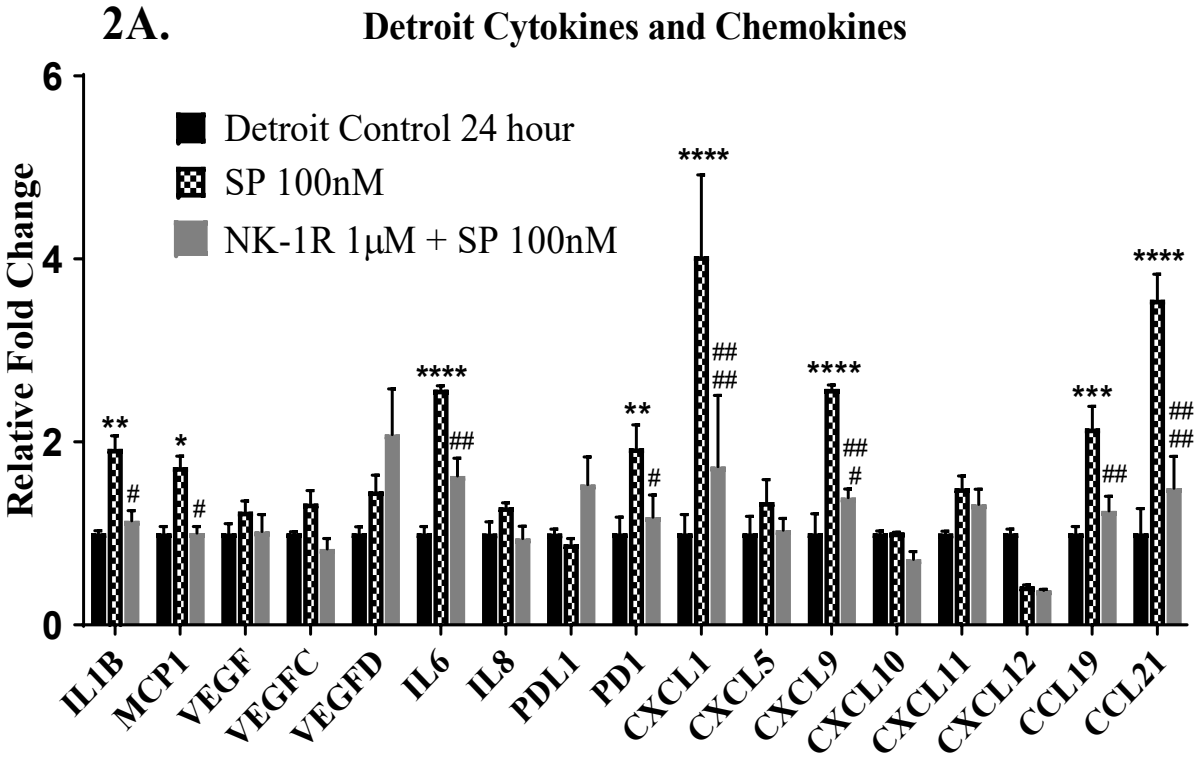
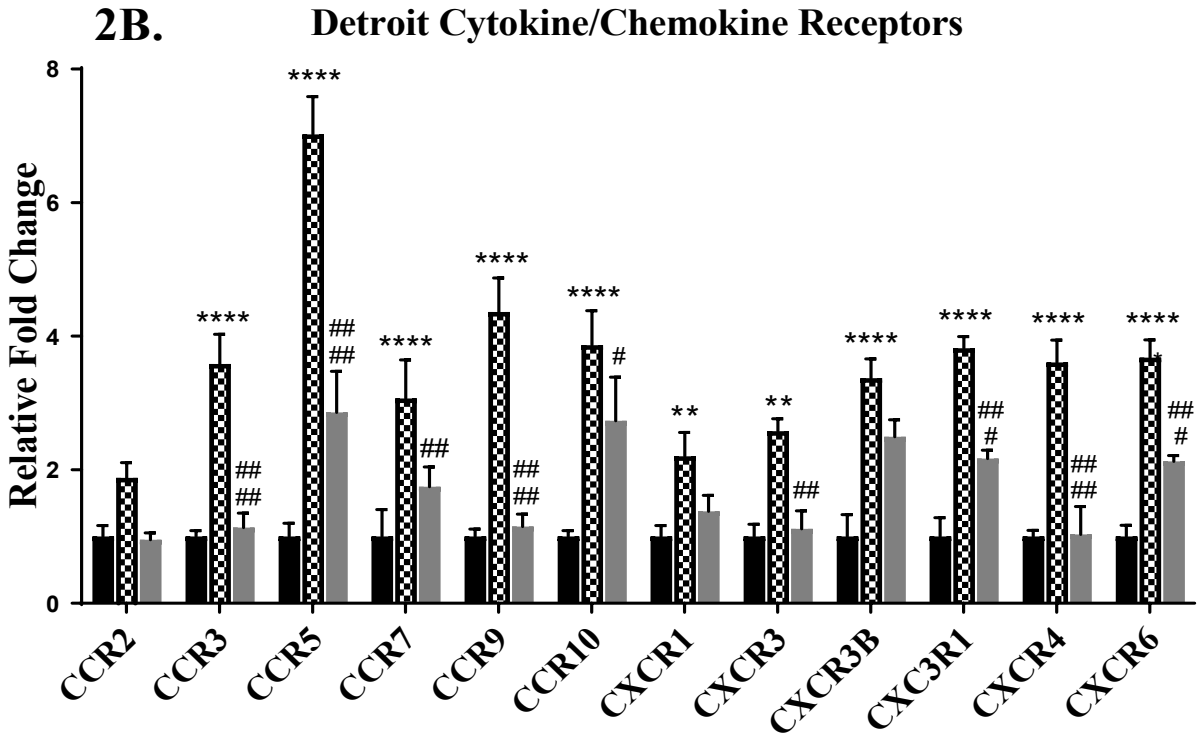


Figure 1: Substance P induced expression and NK-1R inhibition of various cytokines, chemokines, cytokine receptors, chemokine receptors, MMP amd EMT inducing genes in the FaDu cell line. FaDu cells were treated with SP 100nM and with/without NK-1R inhibitor 1μM. mRNA expression of genes were measured for cytokines and chemokines (**Fig. 1A**), cytokine and chemokine receptors (**Fig. 1B**) and MMP and EMT inducing genes (**Fig. 1C**), by RT-PCR analysis. RPL19 was used as the housekeeping gene. Values plotted represent mean ± SEM, n = 3, and Two-way ANOVA was done, followed by Fisher LSD for multiple comparisons. *, **, ***, **** or #, ##, ### and #### indicates $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, $p \leq 0.0001$ as compared to control or SP 100nM, respectively.


2A.
Detroit Cytokines and Chemokines
Detroit Control 24 hour
SP 100nM
NK-1R 1μM + SP 100nM
Relative Fold Change
0
2
4
6
IL1B
MCP1
VEGF
VEGFC
VEGFD
IL6
IL8
PDL1
PD1
CXCL1
CXCL5
CXCL9
CXCL10
CXCL11
CXCL12
CCL19
CCL21


2B.
Detroit Cytokine/Chemokine Receptors
Relative Fold Change
0
2
4
6
8
CCR2
CCR3
CCR5
CCR7
CCR9
CCR10
CXCR1
CXCR3
CXCR3B
CXC3R1
CXCR4
CXCR6

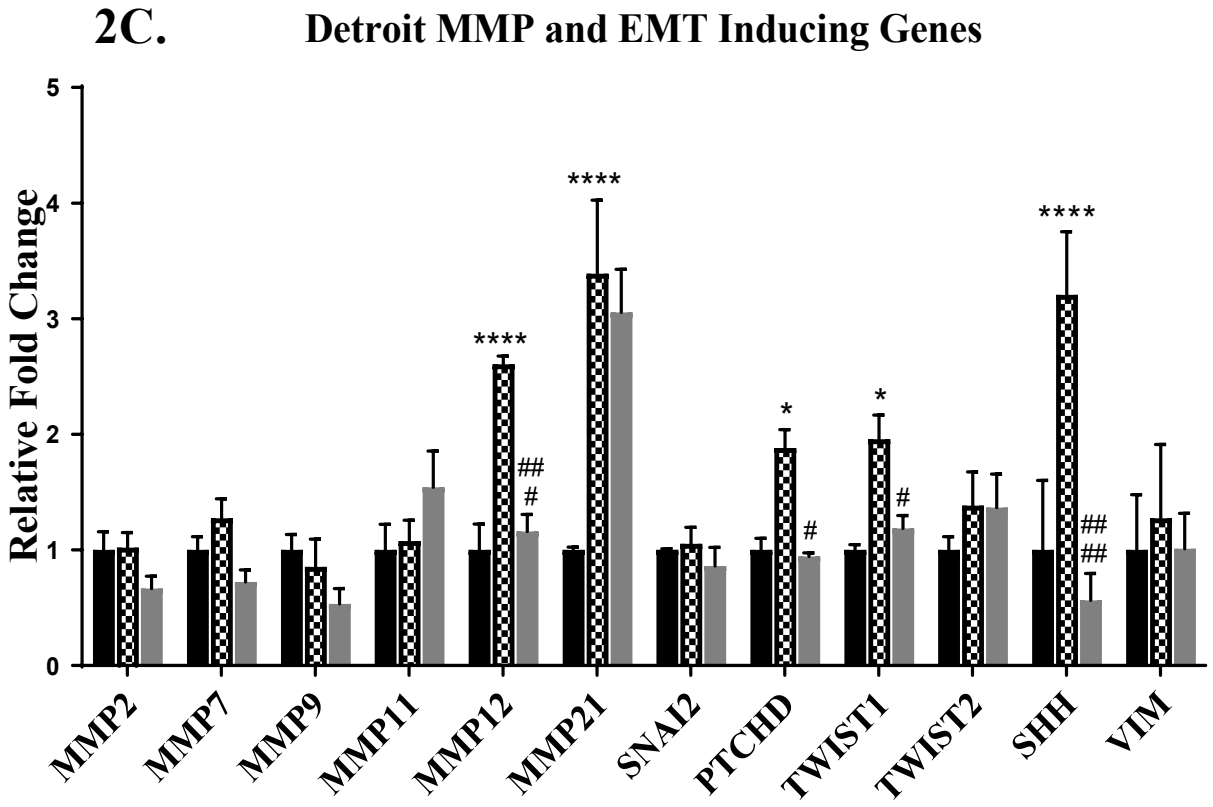
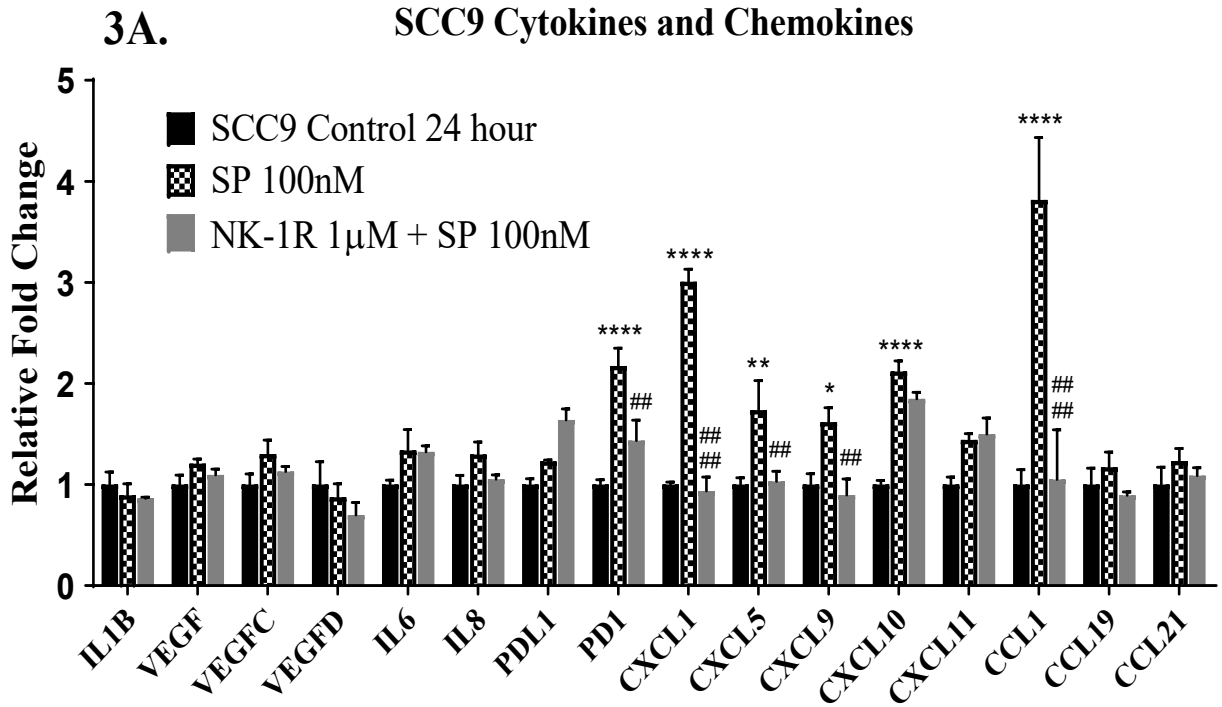
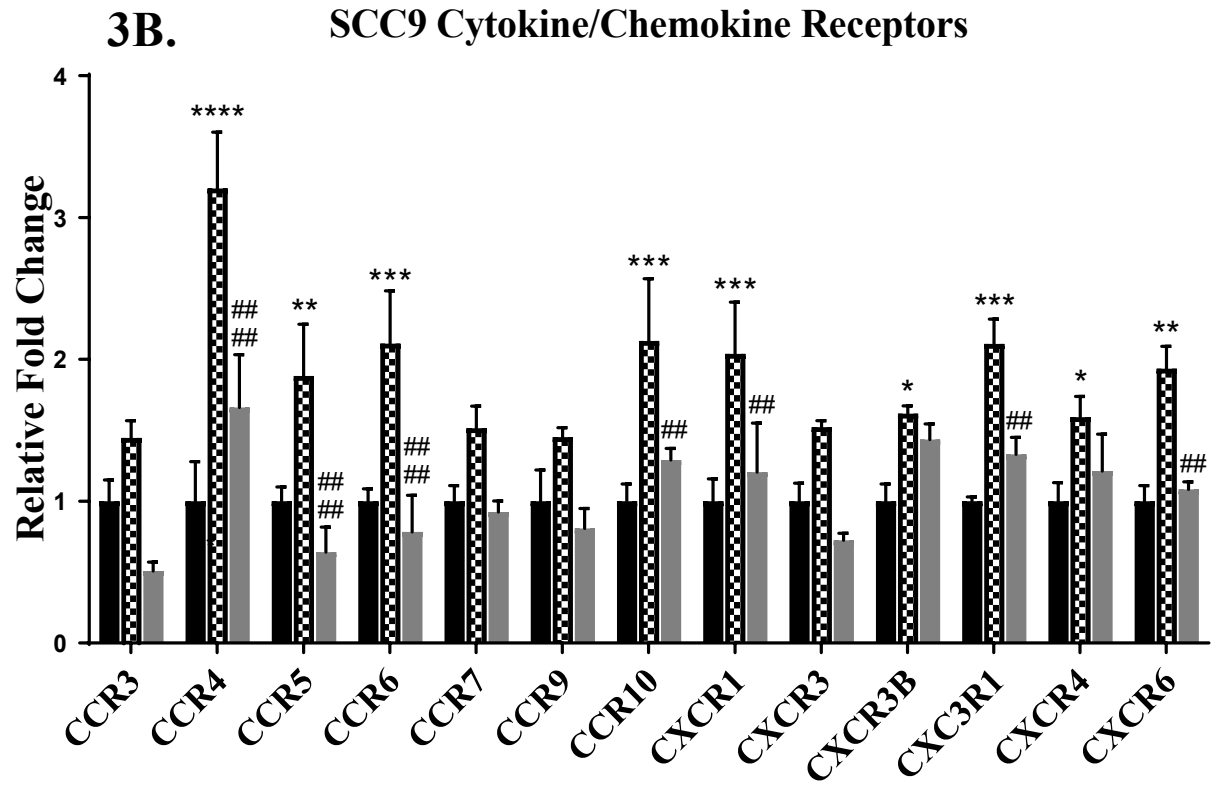


Figure 2: Substance P induced expression and NK-1R inhibition of various cytokines, chemokines, cytokine receptors, chemokine receptors, MMP amd EMT inducing genes in the Detroit cell line. Detroit cells were treated with SP 100nM and with/without NK-1R inhibitor 1μM. mRNA expression of genes were measured for cytokines and chemokines (**Fig. 1A**), cytokine and chemokine receptors (**Fig. 1B**) and MMP and EMT inducing genes (**Fig. 1C**), by RT-PCR analysis. RPL19 was used as the housekeeping gene. Values plotted represent mean ± SEM, n = 3, and Two-way ANOVA was done, followed by Fisher LSD for multiple comparisons. *, **, ***, **** or #, ##, ### and #### indicates $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, $p \leq 0.0001$ as compared to control or SP 100nM, respectively.


3A.
SCC9 Cytokines and Chemokines
SCC9 Control 24 hour
SP 100nM
NK-1R 1μM + SP 100nM
Relative Fold Change
0
1
2
3
4
5
IL1B
VEGF
VEGFC
VEGFD
IL6
IL8
PDL1
PD1
CXCL1
CXCL5
CXCL9
CXCL10
CXCL11
CCL1
CCL19
CCL21


3B.
SCC9 Cytokine/Chemokine Receptors
Relative Fold Change
0
1
2
3
4
CCR3
CCR4
CCR5
CCR6
CCR7
CCR9
CCR10
CXCR1
CXCR3
CXCR3B
CXC3R1
CXCR4
CXCR6

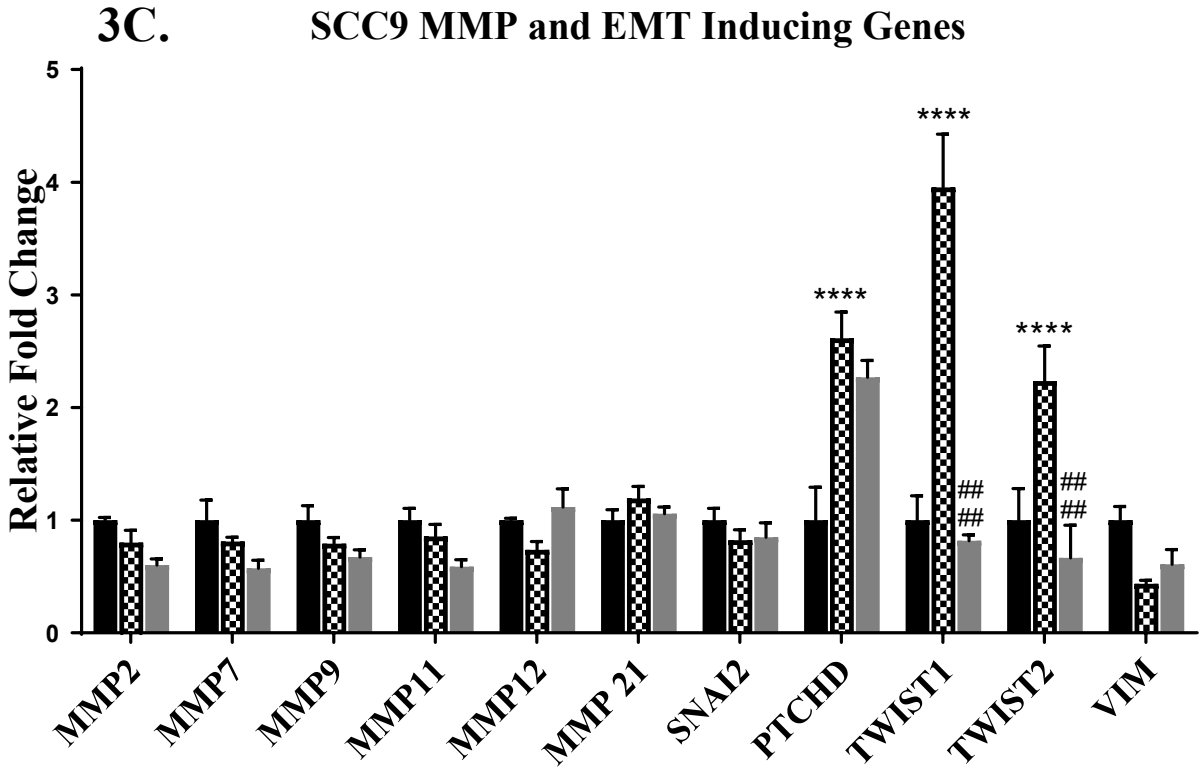


Figure 3: Substance P induced expression and NK-1R inhibition of various cytokines, chemokines, cytokines receptors, chemokine receptors, MMP amd EMT inducing genes in the SCC9 cell line. SCC9 cells were treated with SP 100nM and with/without NK-1R inhibitor 1μM. mRNA expression of genes were measured for cytokines and chemokines (**Fig. 1A**), cytokine and chemokine receptors (**Fig. 1B**) and MMP and EMT inducing genes (**Fig. 1C**), by RT-PCR analysis. RPL19 was used as the housekeeping gene. Values plotted represent mean ± SEM, n = 3, and Two-way ANOVA was done, followed by Fisher LSD for multiple comparisons. *, **, ***, **** or #, ##, ### and #### indicates $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, $p \leq 0.0001$ as compared to control or SP 100nM, respectively.

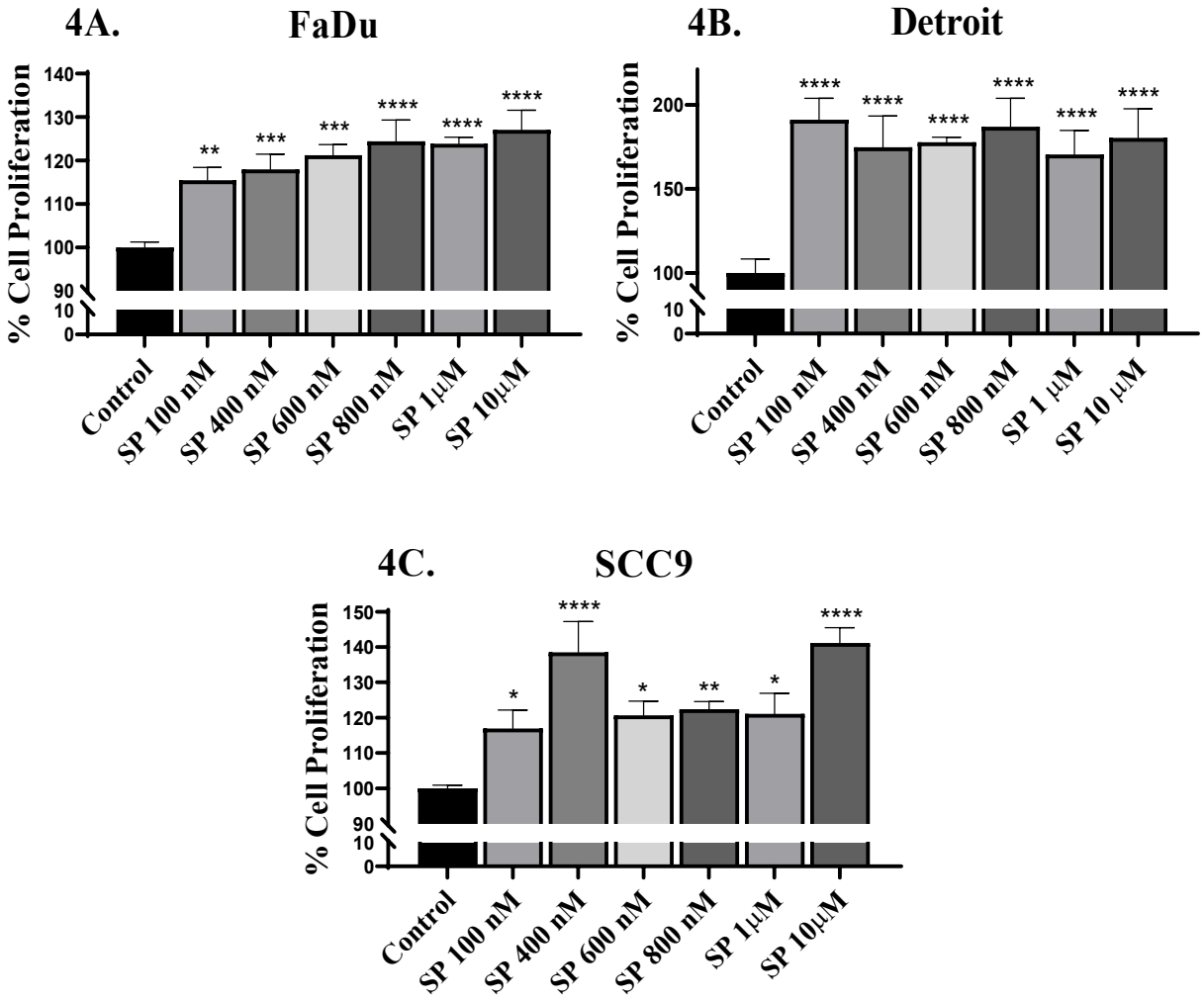
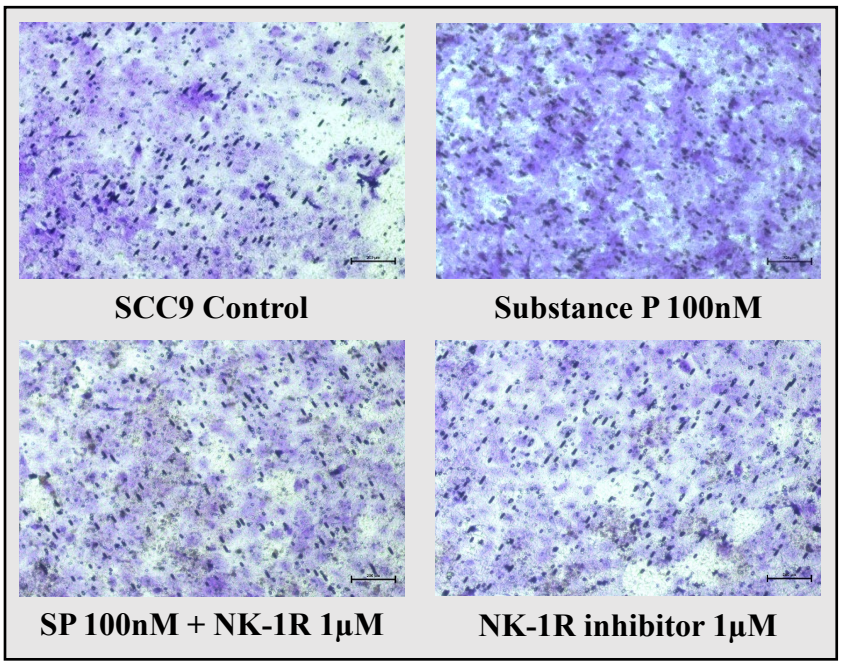


Figure 4: Substance P caused a significant increase in cellular proliferation of HNC cell lines, at various concentrations. HNC cell lines were treated with various concentrations of SP, 100nM - 10μM, for 24 hours. XTT assay was done to measure cellular proliferation on cell lines FaDu (**Fig. 4A**), Detroit (**Fig. 4B**) and SCC9 (**Fig. 4C**). Values represent mean ± SEM, $n \geq 3$, and Two-way ANOVA was done, followed by Fisher LSD for multiple comparison. *, **, ***, **** indicates $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, $p \leq 0.0001$ respectively, as compared to control.

5A.



5B.

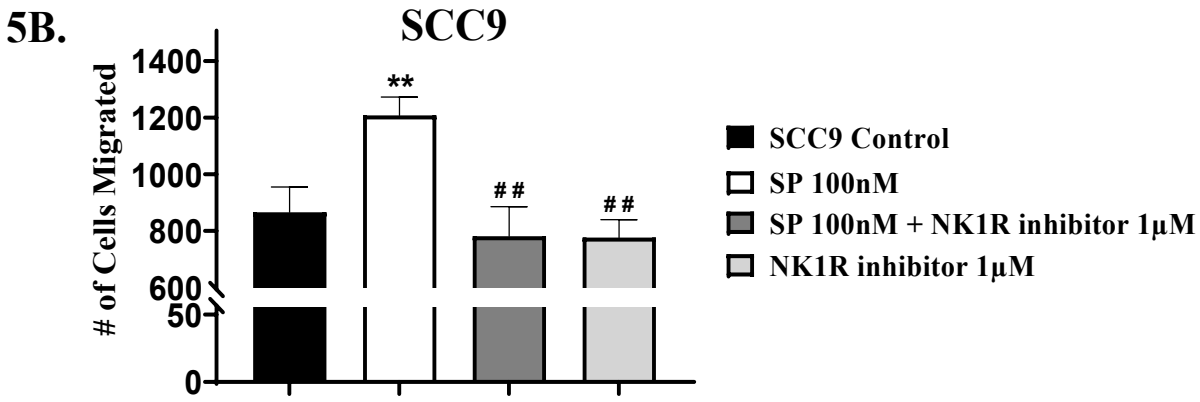


Figure 5: Boyden chamber migration of SCC9 cells was significantly increased by Substance P treatment, while inhibited by NK-1R inhibitor treatment. SCC9 cells were treated with SP 100nM and with/without NK-1R inhibitor 1µM and placed in 8µm pores containing PTE inserts. Cells were allowed to migrate for 24 hours towards complete media. 10x images of inserts were captured and are shown (**Fig. 5A**). Cells were quantified, and the number of cells migrated was plotted on the graph shown in (**Fig. 5B**). Values represent mean ± SEM, n = 5 images, and Two-way ANOVA was done, followed by Fisher LSD for multiple comparison. ** or ## indicates $p \leq 0.01$ as compared to control or SP 100nM respectively.

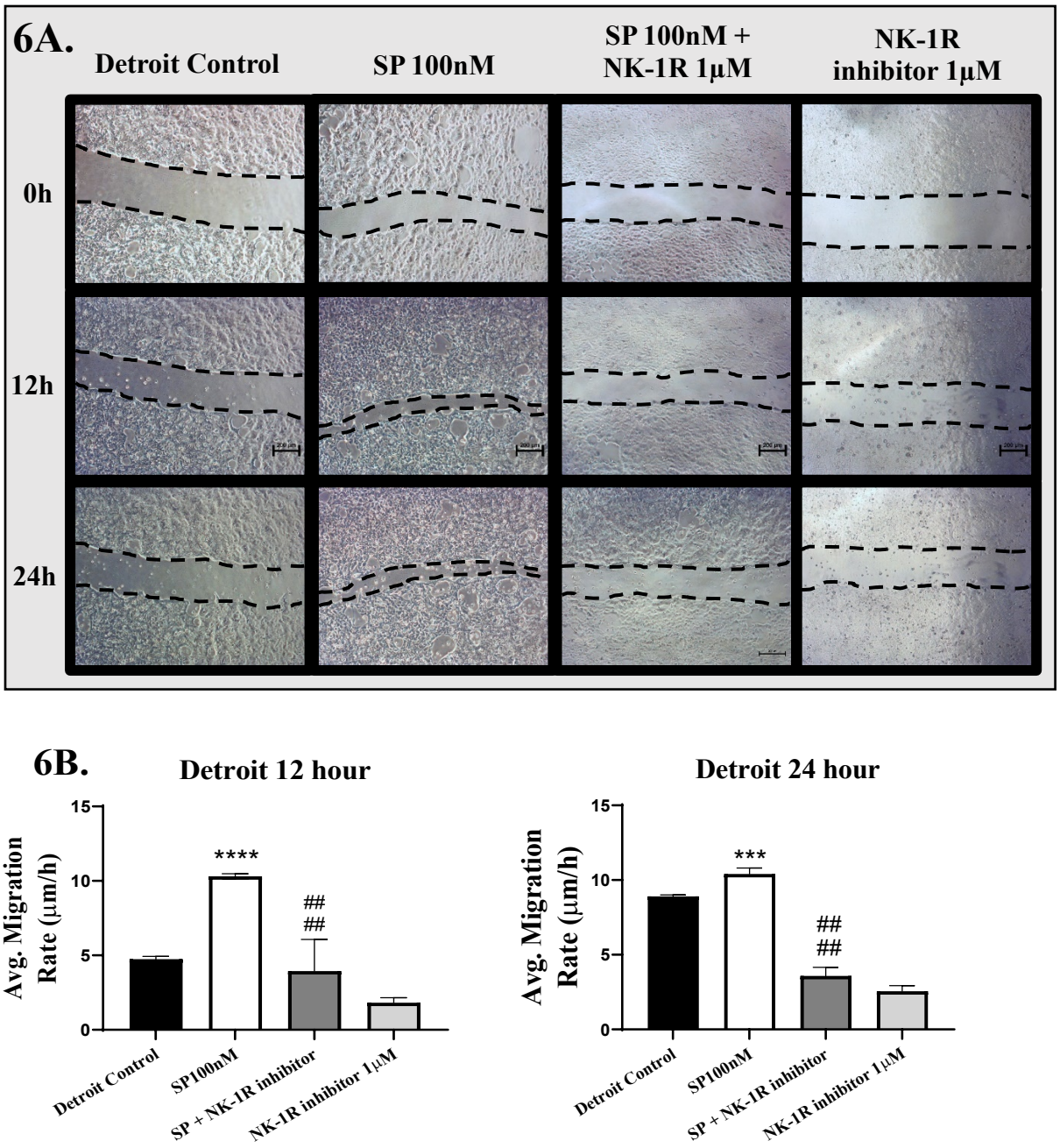


Figure 6: Substance P promotes scratch wound closure of Detroit cells, which is inhibited by NK-1R inhibitor. Detroit cells were treated with SP 100nM and with/without NK-1R inhibitor 1µM. Cells were plated on 12 well plates and a scratch was made in each well. Scratch wound closure was allowed for 24 hours and 4x images were captured at 0, 12 and 24 hours (**Fig. 6A**). Migration rate was measured in µM/hr and plotted (**Fig. 6B**). Values represent mean ± SEM, n = 3 images, and Two-way ANOVA was done, followed by Fisher LSD for multiple comparison. **** or #### indicates p≤0.0001 as compared to control or SP 100nM respectively.

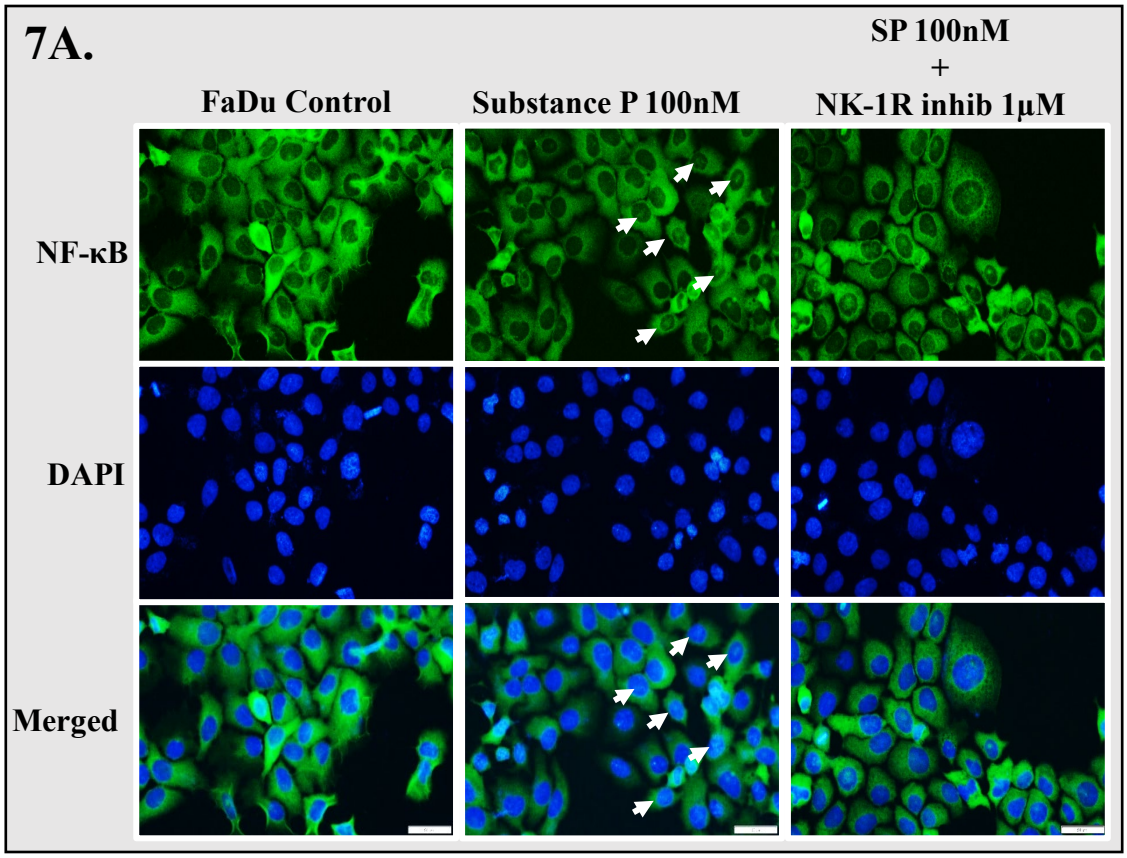


Figure 7: Substance P upregulated NF-κB translocation via Immunofluorescence, which was also abrogated by NK-1R inhibitor in FaDu cells. FaDu cells were treated with SP 100nM and with/without NK-1R inhibitor 1µM. Cells were placed on slides and stained appropriately. 20x images of slides were captured and are shown (**Fig.7A**). The intensity of nuclear NF-κB fluorescence was measured and plotted (**Fig. 7B**). Values represent mean ± SEM, n = 3 images, and Two-way ANOVA was done, followed by Fisher LSD for multiple comparison. ** or ## indicates $p \leq 0.05$, $p \leq 0.01$ as compared to control or SP 100nM respectively.